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Interaction Between Oxidized Hemoglobin and the Cell Membrane: A Common Basis for Several *falciparum* Malaria–Linked Genetic Traits

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ABSTRACT This paper focuses on the interaction between oxidized hemoglobin and the erythrocyte membrane, and its relevance to some *falciparum* malaria–linked genetic traits. We first present the experimental evidence which suggests that the interaction between hemoglobin derivatives and membrane proteins is an important cellular mechanism for the erythrocytes carrying HbS, HbE, HbF, α- and β-thalassemia, and G6PD deficiency. Thereafter, we show how the Hb/membrane interaction might act as primum movens for diverse cellular mechanisms which 1) reduce invasion of erythrocytes by the *falciparum* parasite; 2) impair parasite survival and development within the cell; 3) accelerate infected erythrocyte clearance by phagocytosis. We claim that oxidative stress is the driving force of this process, since highly reactive species (like O_2^- and H_2O_2) mediate the gradual oxidation of Hb to irreversible hemichrome-containing Heinz bodies. We therefore suggest that positing the interaction between oxidized hemoglobin and cell membrane as a common basis for several *falciparum* malaria–linked genetic traits is not only consistent with experimental evidence gathered so far, but provides a new, clearer perspective: the molecular event on which these known protective traits rest. In the last part of the paper we will discuss two case studies which provide further support for the role played by hemoglobin derivatives and membrane proteins: 1) the influence of a cyanogen-rich diet on the distribution of Hbβ*S gene frequencies in Liberia (Jackson [1990] Am. J. Hum. Biol. *2*:521–532); and 2) population data on polymorphisms at the Hbβ and GPX1 loci (Destro-Bisol and Spedini [1989] Am. J. Phys. Anthropol. *79*:217–224).

> Evolution does not produce novelties from scratch. It works on what already exists, either transforming a system to give it new functions or combining several systems to produce a more elaborate one. (Jacob, 1977, p. 1164)

The relationship between malaria and evolution of the human genome has attracted noteworthy interest from physical anthropologists and human geneticists since the 1950s (Haldane, 1949a,b, 1957; Motulsky, 1960; Livingstone, 1971; Luzzatto, 1979), and attention to this topic has continued unabated in the last decade (Livingstone, 1984; Wheaterall 1987; Motulsky, 1989; Hill, 1992; Greene, 1993).

The breadth of experimental, clinical and field research carried out in the past 40 years has led to the paradigm that malaria is the key selective factor in attainment and maintenance of relatively high frequencies of several genetic traits. Three important families of blood polymorphism in man (abnormal

hemoglobins, thalassemias and glucose-6-phosphate-dehydrogenase [G6PD] deficiency) are usually considered among the genetic factors conferring resistance to *Plasmodium falciparum.* Sickling trait is widely taken as a clearcut example of "balanced polymorphism"; the selective advantages conferred to heterozygotes are, in fact, balanced by the disadvantages suffered by homozygotes. The frequency of the mutant gene tends, therefore, to be constant over time, if stable environmental conditions are provided (Livingstone, 1971). Conversely, other malaria-linked genetic traits, like hemoglobin E and C, have been suggested to be in the process of replacing other alleles at the same locus (Das et al., 1975; Cavalli-Sforza and Bodmer, 1971).

It is somewhat paradoxical that while the relationship between malaria and certain genetic traits is widely assumed to be substantiated in research papers as well as in textbooks, the mechanisms underlying such adaptations are not clearly understood. As we will see later, such an "impasse" does not arise from the lack of scientific effort in the field. Nonetheless, it emerges clearly that the rapid accumulation of data over the last 40 years has not been paralleled by a striking advance in our understanding of the phenomenon. This situation is hardly unexpected if one considers how many factors, other than genetic ones, are active in determining the extent and pattern of malaria infection in human populations: the strain of *falciparum,* habits in humans and mosquito, climatic, ecological and other environmental conditions, topography and soil, as well as mosquito-control strategies. As a matter of fact, the complexity of the overall phenomenon (malaria) challenges our ability to develop experimental designs and theoretical models aimed at understanding any single etiological component. A possible strategy to overcome this intricate situation could be provided by "the application of new perspectives, that is the introduction of novel frameworks, concepts, or ideas. New perspectives applied to existing data promote new insights and pave the way for new directions of thought and research . . ." (Stuart-Macadam, 1992, p. 39). Such a new perspective can be detected in preexisting knowledge too, provided that there exists a significant degree of analogy between the already understood phenomenon and that needing to be explored: ". . . in the history of sciences, important advances often come from bridging the gaps. They result from the recognition that two hitherto separate observations can be viewed from a new angle and seen to represent nothing but separate facets of one phenomenon . . ." (Jacob, 1977, p. 1162).

We will here show that a new perspective for understanding how some erythrocytic genetic traits protect erythrocytes against *falciparum* malaria is offered by the interaction between oxidized derivatives of hemoglobin and proteins of the erythrocyte plasma membrane. The importance of such an interaction for senescence in uninfected erythrocytes is well established (Chiu and Lubin, 1989; Hebbel, 1991; Low, 1991), but its relevance to the processes acting in erythrocyte genetic adaptations to malaria has not been investigated so far.

The core of this paper is divided, although not explicitly, into three sections. After having introduced some general concepts, we will present the experimental background which makes the interaction between hemoglobin derivatives and membrane proteins a plausible mechanism for several malaria-linked protective genetic traits. Thereafter, we will show how different malaria-protective cellular events may be the final effect of processes initialized by the Hb/membrane interaction. Finally, we will organize the evidence presented in a coherent framework, in which the cause-effect relationships between the different cellular events are specified.

In the last part of the paper we will discuss two case studies which can provide further support to the role played by hemoglobin derivatives and membrane proteins: 1) the influence of sublethal cyanogens included in the diet on the distribution of Hbβ*S gene frequencies in Liberia (Jackson, 1990); 2) population data on polymorphisms at the Hbβ and GPX1 loci (Destro-Bisol and Spedini, 1989).

RISE AND DIFFUSION OF *FALCIPARUM* MALARIA

It is generally agreed that the process leading to the evolution of the four species of plasmodia relevant to humans (*Plasmodium falciparum, P. malariae, P. ovale* and *P. vivax*) began in the early Tertiary age (Garnham, 1966), but it was only much later, in the early Pleistocene, that the first ancestors of *P. falciparum* emerged (Bruce-Chwatt, 1988; Zulueta, 1994). Besides being the most recent plasmodial acquisition for human populations, *P. falciparum* is also the most highly pathologic *Plasmodium* for our species. According to a recent and accepted view, the parasite would have evolved from avian plasmodia (Waters et al., 1991, 1993). The geographical location of *falciparum* evolution and dispersal is controversial: Bruce-Chwatt (1965), Mattingly (1976) and more recently Knell (1991) indicated the tropical rainforests of Africa, in contrast to Coatney et al. (1971), who suggested the tropical rainforests of Southeast or South-central Asia. Wherever *P. falciparum* spreading may have started, temperature conditions allowing for the development of the parasite and breeding of some of its most effective vectors were established in southern Europe and the Near East only at the end of the Würm glaciation, approximately 10,000 years ago (Zulueta, 1994).

After temperature conditions, the second main limiting factor for the spreading and maintaining of *falciparum* infection in ancient populations was, certainly, the establishment of a high and constant rate of infection for humans and mosquitos. These conditions imply a certain level of population density of human groups settled in stable communities. It is unlikely that the latter requirement could have been attained prior to the introduction of the domestication of plants for systematic food production (Boyd, 1949). At the same time, some alterations of the local environment connected to agricultural practices created suitable ecological conditions for some *Anopheles* species, such as small temporary pools of water exposed to direct sunlight in deforested areas, which offer an optimal medium for *A. gambiae* breeding (Livingstone, 1958; Coluzzi, 1994).

It is thought that wild cereals were brought under domestication in the Near East by 7000 BCE and were probably introduced into Europe not later than 6000 BCE (Ammermann and Cavalli-Sforza, 1984). Archeological and linguistic evidence suggests that food production did not become widespread in North Africa until about the sixth millennium BCE, but it seems unlikely that the first agricultural practices were accompanied by establishment of stable settlements (Phillipson, 1985). As to tropical Africa, some authors envisage that agriculture was introduced only when forest clearance was made possible by the advent of iron (Livingstone, 1958; Ehret, 1984). Accordingly, the onset of agriculture in this area should not predate 1000 BCE. To support a scenario of relatively recent diffusion of the parasite in this last area, Coluzzi et al. (1985) found evidence that *A. gambiae,* the most effective *falciparum* vector, underwent a period of intense evolution in recent times, a phenomenon that probably coincides with agricultural development given the highly anthropophilic habit of this mosquito species.

Interesting contributions to the reconstruction of *P. falciparum*'s past epidemiology have also been provided by paleopathologists, who based their conclusions on the distribution of porotic hyperostosis in ancient human populations. This is a pathological condition resulting from erythroid hyperplasia affecting the skull vault and the orbital roof (see Stuart-Macadam, 1992, for a recent view on this topic). Based on the assumption that thalassemia in association with *falciparum* malaria is responsible for porotic hyperostosis found in some ancient skeletal samples from Anatolia, Greece and Cyprus, Angel (1966) hypothesized that mutation producing resistance to *falciparum* malaria antedate the seventh millennium BCE and have an eastern Mediterranean origin. However, this conclusion remains controversial given the complex etiologies of this pathological sign (Ascenzi et al., 1979). Application of DNA sequence analysis has recently proved a valuable means to detect

hyperostotic manifestations due to thalassemia (Filon et al., 1995).

Dealing with the early history of malaria, we cannot overlook those reconstructions based on the analysis of historical sources. Though important and useful, this approach has also an intrinsic limitation: the lack of knowledge, until the late 19th century, of the etiologic role of the different *Plasmodia* species. Diagnosis therefore rests on the analysis of recorded signs and symptoms, but such information is often inadequate for a reliable distinction between *vivax* and *falciparum* malaria, and sometimes between malaria and other febrile conditions.

The analysis of the Ebers papyrus and of the hyeroglyphs at Dendera seems to indicate an appearance of malaria in the Nile valley prior to 1500 BCE (Helck and Otto, 1982), suggesting that malaria appeared earlier in the African continent than in the eastern Mediterranean. Malaria is also claimed to be one of the most important factors in the fall of the Hindu civilization in the second millennium BCE (Zulueta, 1994). According to Grmek (1988), a historical report of *falciparum* malaria may be traced to the first and third books of *Epidemics* by Hippocrates (born 460 BCE). This claim is questioned by Zulueta (1994), who suggested that *falciparum* malaria was rare, if not absent, in ancient Greece. In any case, the two authors substantially agree with Sallares (1991) that *falciparum* malaria represented a serious health problem for ancient Romans (Grmek, 1994). The disease was already widespread in Europe during the Middle Ages and lasted until the 19th century (Sigerist, 1985), but *falciparum* as an endemic disease remained limited to the southern area of the continent. The *falciparum* parasite was presumably absent in pre-Columbian America (Effertz, 1909; Dunn, 1965). Malaria was endemic in the northern lands of Australia before its eradication in 1962 (Marshall, 1993). The presence of the disease in this area predates the European colonization, and it is probably due to the introduction of *Plasmodia* from New Guinea. Conversely, the disease was introduced in the Solomon Islands and outlier atolls of Papua New Guinea only after the European contact in the 19th century (Marshall, 1993).

As to its present-day distribution, the *falciparum* parasite is still dispersed in a large geographic area including Southwest Asia (e.g., India, Pakistan) Southeast Asia (e.g., Indonesia), Australasia (e.g., New Guinea and Melanesia) and Central and South America. As regards to Africa, *falciparum* malaria is widespread south of the Sahara, with malaria-free zones only in the southern part of the continent (Botswana, South Africa, and Namibia) and at high altitudes. Although one must take into account the remarkable differences from one area to another in transmission patterns, immunity and clinical effects, mainly related to ecological diversity (see Carnevale et al., 1984), *falciparum* malaria attains a great epidemiological relevance in a great part of sub-Saharan Africa mainly because: 1) the presence of climatic conditions favorable to the sporogonic development of the most effective *P. falciparum* vector combination, provided by *A. funestus* and the *A. gambiae* complex (comprising the *A. gambiae* s.s. and *A. arabiensis*); 2) the impact of man-made ecological changes. The combination of these factors results in the highest potential for malaria transmission in the world (Coluzzi, 1994), as witnessed by the fact that more than 80% of the estimated 120 million clinical cases worldwide come from sub-Saharan Africa.

The increasing resistance to antimalarial drugs and DDT by the *falciparum* parasite and mosquito vector, respectively, hinders significant progress in eradicating this disease (Wernsdorfer, 1994). Drawbacks have also been encountered in the development of a vaccine, probably because of the antigenic diversity during different stages and among diverse parasite strains (Cox, 1991).

PLASMODIUM FALCIPARUM LIFE CYCLE

P. falciparum shows so many differences in morphology, physiology and life cycle from other human-specific members of its genus as to be at one time also referred to as another genus (Bray, 1958) or, more recently, subgenus, called in both cases *Laverania*. However, as for the other human specific plasmodia, the life cycle is complex and involves both mosquito and vertebrate hosts. Male and female gametocytes are first in-

gested following the bite of an infected human by a female mosquito, in which stomach sexual reproduction of the parasite occurs. Thereafter, the zygote migrates through the stomach wall, where it undergoes asexual division to form numerous sporozoites. These migrate to the salivary glands, and are injected into the human bloodstream when a new blood meal is taken. Sporozoites quickly circulate to the liver where they develop further in the hepatocytes, giving rise to the exoerythrocytic stages. Each exoerythrocytic schizont releases about 30,000 new merozoites every 48–72 hours that subsequently invade the erythrocytes. In the erythrocytic cycle, the earliest form after invasion (ring form) develops in trophozoites of increasing sizes. Meanwhile, excrescences (knobs) appear on the outer surface of red cells. Knobs mediate the attachment of the infected erythrocytes to the venular vascular endothelium of internal organs such as the brain, heart, placenta, spleen and bone marrow. The sequestration of infected erythrocytes usually leads to their disappearance from the peripheral circulation after 24 hours from infection. The life cycle is completed by the generation of schizonts that undergo segmentation, each giving rise to eight to 24 intraerythrocytic merozoites. Such forms are released in the bloodstream after the rupture of the cell. The characteristic fevers and chills which are the symptoms of the infection originate from the periodic rupture of erythrocytes by the parasite. The development of the gametocytes usually takes place in the inner organs, and mature gametocytes generally appear in the peripheral blood about 10 days after the initial invasion of the erythrocytes.

Once an individual is infected by *P. falciparum,* there is a range of possible outcomes, including death. Clinical manifestation correlates with the density of parasitaemia and immunological status of the host and depends mainly on the distribution of the parasites in host organs and tissues. Cerebral malaria, for example, the most notorious form of severe malaria, is associated with sequestration of the parasite in blood vessels of the brain.

PLASMODIUM FALCIPARUM AS A SELECTIVE AGENT

In addition to its epidemiological relevance, the *falciparum* parasite has some features that make it one of, if not the, most important selective agent for human evolution. Although the exact timing of the spread of *falciparum* may be questioned, there are some well-established reasons to assume that both the number of generations since parasite emergence and the intensity of *falciparum* selective pressure have been sufficient to produce appreciable evolutionary consequences in the gene pools of many human populations.

First, the mortality rate of the *falciparum* parasite has been very high from the early spreading of the parasite to the present. This can be easily inferred if one considers a recent estimate: *P. falciparum* causes between one half and two million deaths per year in sub-Saharan Africa alone (see Hill et al., 1991).

Second, it can be reasonably assumed that *falciparum* malaria remained endemic in most parts of those areas where the parasite spread several thousand years later. In such an epidemiological condition, exposure of humans to the parasite leads to the acquisition of specific immunity, which usually develops in 2–5 years. At the same time, children born to immune mothers remain relatively protected for a period between 3 and 6 months following birth. Life-threatening malaria complications are thus generally restricted to the prereproductive period, within the first 5 years of age; in accordance with the estimates by Greenwood et al. (1987), in the Gambia about 1% of children die each year by malaria. Constant exposure to the parasite, generation after generation, has produced selective effects that are particularly constant and cumulative over time (Livingstone, 1971). Therefore, appreciable evolutionary consequence may be expected when a population has been subjected to endemic malaria for many generations.

Conversely, a lower selective effectiveness may be expected in epidemic malaria, where all ages are almost equally affected. Although long and disastrous, each epidemic produces an effect on gene frequencies com-

parable to that of a single generation of selection, despite the mortality rate being greater than in endemic malaria. Moreover, an epidemic is by definition a discontinuous event and, therefore, its selective consequences cannot accumulate over time. Accordingly, the number and frequencies of protective malaria traits are lower in areas where malaria was mainly epidemic (Livingstone, 1971).

CURRENT UNDERSTANDING OF GENETIC ADAPTATIONS TO *FALCIPARUM* MALARIA

The first formulation of the "malaria hypothesis"—in which the evolutionary affirmation of several mutant genes is seen as a consequence of their capacity to protect against malaria parasites—is usually referred to a lecture held in 1949 by J.B.S. Haldane at the Seventh International Congress of Genetics. Although thalassemia triggered Haldane's hypothesis, from a historical point of view it must be acknowledged that protective capacity against malaria by the sickling trait was perceived earlier (Beet, 1946, 1947).

The updated list of the genetic traits with a presumable or probable protective capacity against *P. falciparum* contains several abnormal hemoglobins, enzymatic defects, erythrocyte and leucocyte antigens, and cytoskeleton abnormalities (Table 1). Absent in red blood cells, HLA angtigens presumably mediate a protection against the liver-stage malaria parasites. All the remaining traits are, conversely, involved in the defense against the erythrocytic stages of *P. falciparum*.

The geographical coincidence between *falciparum* malaria endemicity and the distribution of HbS, thalassemias and G6PD deficiency provided the stimulus for the formulation of the "malaria hypothesis," as well as its first support (Allison, 1954a,b,c; Motulsky, 1960; Siniscalco et al., 1966). Several exceptions to this relationship have, however, been reported (Brown, 1981; Bowman and Murray, 1990). It is clear, however, that analyses of geographical association between parasite and mutant erythrocytes cannot by themselves prove or disprove the malaria hypothesis. In fact, the geographical approach could provide an effective means of malaria hypothesis testing only with assumptions too drastic to be met by most human populations: stable ecological and demographic conditions for hundreds of years combined with absence of significant levels of migration or admixture and, finally, nonrandom mating (Bowman and Murray, 1990). Furthermore, it is required that transmission and expression of each genetic factor are exempt from the influence of other inherited traits and/or selection factors. Significant advances in this field may, however, be expected from the application of molecular genetic techniques. This is exemplified by recent studies of globin genes that have disclosed the possibility of distinguishing the patterns of HbS and thalassemia variation created by selection from those due to population migration and genetic drift (Flint et al., 1993). As emphasized by the authors, such a possibility is limited to those populations for which malaria prevalence rates, population structure and prehistory are reliably known.

In order to obtain more stringent proof, some authors undertook the comparison of

TABLE 1. Updated list of genetic traits with a presumable protective role against falciparum *malaria*

	Reference
Abnormal hemoglobins	
HbS	Allison, 1954a,b,c
HbC	Thompson, 1962
HbE	Flatz et al., 1964
Hereditary persistence of HbF	Pasvol et al., 1977
α-thalassemia	Oppenheimer et al., 1984
β-thalassemia	Chernoff, 1959
Erythrocyte enzymatic defects	
G6PD	Allison, 1960
Pyridoxal-kinase	Martin et al., 1978
Pyridoxine-phosphate Oxidase	Anderson et al., 1993
Glutathione reductase	Anderson et al., 1993
Erythrocyte surface antigens	
En(a-) RBCs	Pasvol et al., 1982a
S-s-U negative RBCs	Pasvol et al., 1982b
Tn RBCs	Pasvol et al., 1982b
Wr^{b-} RBCs	Pasvol et al., 1982b
Gerbich negative RBCs	Nagel, 1990
Leucocyte surface antigens	
HLA-Bw35	Hill et al., 1991
HLA DRB1*1302-DQB1*0501	Hill et al., 1991
Erythrocyte cytoskeleton abnormalities	
Ovalocytosis	Baer et al., 1976
Hereditary spherocytosis	Nagel, 1990
Elliptocytosis	Nagel, 1990

TABLE 2. Refractoriness to develop severe malaria (severe malarial anaemia and cerebral malaria) in some malaria-linked genetic traits

	Reference
G6PD deficiency[1]	Ruwende et al., 1995
HbE	So Satta et al., 1970; Goueffon and du Saussay, 1969
HbS[2]	Livingstone, 1971; Hill et al., 1991
HLA-Bw53 antigen[3]	Hill et al., 1991
HLA DRB1*1302-DQB1*0501 haplotype[4]	Hill et al., 1991
Ovalocytosis heterozygotes	Genton et al., 1995
α-thalassemia[5]	Yates et al., 1993
β-thalassemia	Willcox et al., 1983

[1] Ruwende et al. (1995) showed a 46–58% reduction in risk of severe malaria for female heterozygotes and male hemizygotes for the common African form of G6PD deficiency.
[2] Hill et al. (1991) showed a 92% reduction in risk of severe malaria for HbAS subjects.
[3] Hill et al. (1991) showed a 41% reduction in risk of severe malaria for HLA-Bw53 carriers.
[4] As regards to the carriers of the HLA DRB1*1302-DQB1*0501 haplotype, Hill et al. (1991) showed a reduction in risk (64%) only for severe malaria anemia, while protection from cerebral malaria was seen but failed to reach statistical significance. Furthermore, the protection was more pronounced in homozygotes than in heterozygotes.
[5] Protection was observed in α-thalassemia homozygotes.

TABLE 3. Impaired growth in vitro of the falciparum *parasite in some malaria-linked genetic traits*

	Reference
G6PD deficiency[1]	Friedman, 1979; Roth et al., 1983; Kamchonwongpaisan et al., 1989
HbC[2]	Friedman et al., 1979; Olson and Nagel, 1986
HbE[3]	Nagel et al., 1981; Vernes et al., 1986
HbF[4]	Pasvol et al., 1977a; Friedman, 1979
HbS[5]	Luzzatto et al., 1970; Friedman, 1978
α- and β-thalassemia[6]	Friedman, 1979; Brockelman et al., 1987
Ovalocytosis[7]	Kidson et al., 1981; Hadley et al., 1983

[1] Friedman (1979) used erythrocytes containing the common African form of G6PD deficiency, whereas Roth et al. (1983) used erythrocytes containing the Mediterranean form of G6PD deficiency. The experiments by Kamchonwongpaisan et al. (1989) involved exposure of erythrocytes to hydrogen peroxide.
[2] Olson and Nagel (1986) observed impaired release of merozoites.
[3] Vernes et al. (1986) observed maximum impairment of parasite growth in oxygenated conditions (20% O_2).
[4] Pasvol et al. (1977a) and Friedman (1979) observed that retardation in parasite growth disappears at low O_2 (5%).
[5] Friedman observed maximum impairment of parasite growth at low O_2 (3%) after 2 days of normal growth at 17% O_2.
[6] The experiments by Brockelman et al. (1987) were carried out in a culture medium containing a dilute amino acid concentration.
[7] Kidson et al. (1981) and Hadley et al. (1983) observed a reduced erythrocyte invasion by the malaria parasites.

parasitological markers of malaria disease between normal patients and carriers of genetic abnormalities (for a review see Bowman and Murray, 1990). Unfortunately, these studies often yielded controversial or inconclusive results. This is easily explained by the fact that pharmacological treatment, coexistence of different malaria protective traits in the same populations and other factors influencing parasitological and clinical parameters are a potential source of confusion in interpretation of the final results. According to Greene (1993) additional problems may derive from a biased experimental design.

It has been observed that erythrocytic *P. falciparum* rates and densities tend to be lower among HbAS and G6PD deficient subjects (Livingstone, 1971; Greene, 1993), although there is some evidence to contradict this conclusion (Bowman and Murray, 1991). The reduction of parasitemia in heterozygotes for a band 3 deletion seems at present less controversial (Serjeantson et al., 1977; Cattani et al., 1987; Foo et al., 1992).

More encouraging evidence has been provided by analyses of clinical markers. An almost complete refractoriness to develop severe malaria has been repeatedly observed in HbAS subjects (Livingstone, 1971; Hill, 1991), and there is compelling evidence that several other genetic traits also confer such a protection, though with a lower effectiveness than HbS (Table 2).

After the pioneering work by Trager and Jensen (1976), several authors have investigated the growth and course of infection using in vitro erythrocytic culture systems (Friedman, 1978; Pasvol et al., 1978; Roth et al., 1978). Impaired growth of the parasite was observed in almost all mutant erythrocytes (Table 3), but in some cases (e.g., thalassemias, G6PD deficiency) the phenomenon was observed only after an increased oxidative stress or a decreased concentration of certain amino acids (see Nagel, 1990).

Further valuable insights have been provided by the introduction of techniques which make coculturing erythrocytes with peripheral blood monocytes possible (Bunyaratvey et al., 1986). It has thus been observed that infected erythrocytes carrying

HbS, HbE, G6PD Mediterranean deficiency and thalassemias are subjected to enhanced phagocytosis (Allison and Eugui, 1983; Luzzatto and Pinching, 1990; Bunyaratvey et al., 1986; Arese et al., 1994). Recently, Arese and coworkers (1991, 1994) obtained results that might clarify how differential phagocytosis leads to protection from the parasite. They observed that phagocytosis of G6PD deficient infected erythrocytes already takes place in the ring form, a stage in which there is no accumulation of hemozoin, a malarial pigment produced by the digestion of host hemoglobin by the parasite. As hemozoin is responsible for the poisoning of macrophages (Schwarzer et al., 1992), it follows that phagocytosis in the ring stage may result in more efficient removal of infected erythrocytes.

The recent introduction of transgenic and mutant animal models now promises significant advances in understanding the role of the immune system. In particular, this approach enables the study of the role of the reticuloendothelial system in the recognition of infected erythrocytes under well-controlled experimental conditions. Important results have been already achieved by Shear et al. (1993, 1994), who observed that in transgenic β*S mice splenectomy reverses completely the protection against *P. chabaudi adami* infection.

In sum, there is at least sufficient evidence to accept the validity of the malaria hypothesis for several genetic traits (HbS, HbC, HbE, HPHF, α- and β-thalassemia, G6PD deficiency, Southeast Asian ovalocytosys, HLA antigens), while for others (blood group antigens, enzymatic deficiencies other than G6PD) the evidence is at best circumstantial. Some aspects of the first group also need better understanding too, such as the relevance of environmental factors and the combined effect of different traits on the progression of malaria (Murray et al., 1978; Nurse, 1979; Jackson, 1990; Bowman and Murray, 1990).

These and other related issues cannot be clarified until we understand how erythrocytes might protect themselves from *falciparum* infection. In fact, although several cellular events have been invoked to explain the protection against *falciparum* malaria, it is still unclear how they may be triggered in the course of the interaction between *P. falciparum* and the cell. At the end of 1970s some authors tried to fill this gap, suggesting that malaria protective traits may impact *Plasmodium* adversely by placing an increased oxidative stress within the infected erythrocytes (Friedman, 1978; Eckman and Eaton, 1979; Etkin and Eaton, 1975). However, they were unable at the time to identify the molecular basis of the relevant mechanisms and to indicate the stage(s) of parasite invasion or proliferation in which the oxidative mechanisms could be at work.

A NEW PERSPECTIVE ON GENETIC ADAPTATION TO *FALCIPARUM* MALARIA

The rationale of our approach is that the erythrocytes of those individuals who are simultaneously protected against the parasite and exempt from significant pathological manifestations comply with two distinct demands: the maintenance of an adequate functioning of the complex "erythrocyte" machine and the introduction of important cellular perturbations, such as those underlying the protection against *P. falciparum* proliferation. To achieve the new objective without compromising other important cellular functions, the most parsimonious strategy, in evolutionary terms, would be that of applying a mechanism already at work in a physiological context and, therefore, well harmonized within the biochemical pathways of the cell. Such a mechanism should be flexible enough to display two opposite behaviors depending upon the erythrocyte infection status; in uninfected cells, we expect that the ultimate consequences of such a mechanism would be adequately compensated by other biochemical pathways; conversely, in presence of *P. falciparum* we expect that the mechanism may be activated to such an extent as to produce significant effects on metabolism and survival of the parasite/erythrocyte unit.

In the following section of the paper we will explore the possibility that such a mechanism could be provided by the interaction between hemoglobin and the erythrocyte membrane.

Hemoglobin oxidation, erythrocyte membrane and cellular senescence

The specific function of human erythrocytes is the transportation of oxygen from the lungs to the tissues. In order to accommodate more O_2 transporting protein (hemoglobin) and to allow greater flexibility in the passage of the cell through narrow capillaries, human mature erythrocytes lack mitochondria and nuclei. The unavailability of genetic material and of energy-producing compartments gives the human erythrocyte the unique characteristic of a finite lifespan of approximately 120 days.

Besides being responsible for oxygen transport to the tissues, hemoglobin may undergo oxidative processes and play an important role in the process of red cell aging. Reversible binding of O_2 in vivo is accomplished by hemoglobin with the heme iron in reduced (FeII) state. Oxyhemoglobin autoxidizes at a rate of 3% per day to yield methemoglobin (met-Hb, hemoglobin with heme in the ferric form, FeIII) and superoxide anion (O_2^-), which is converted into H_2O_2 by the enzyme superoxide dismutase (Moya et al., 1985). Further oxidation of hemoglobin may be promoted by hydrogen peroxide produced in the previous reaction and anions such as Cl^- (Wallace et al., 1982; Giulivi and Davies, 1990). Thus, following oxidation and denaturation met-Hb molecules may be converted into hemichromes (HCRs), a group of pigments which may be either reversibly (rHCRs) or irreversibly (iHCRs) oxidized. The latter can aggregate to form Heinz bodies (Rachimelwitz, 1975). Hemoglobin's tendency to oxidize is, however, effectively counteracted in normal erythrocytes by the reducing systems, mainly based on the pentose-phosphate pathway. The concentration of met-Hb in normal erythrocytes is maintained at a steady-state level of about 1%. However, being incapable of de novo protein synthesis, erythrocytes cannot replenish antioxidant systems and the capacity of fighting against Hb oxidation is progressively lost in the course of the 120 days of erythrocyte life. Since oxidation of the molecule is paralleled by an increased capacity of binding irreversibly to the inner surface of the erythrocyte membrane, aging of the cell is accompanied by an increase in membrane-bound Hb (Peisach et al., 1975). From the knowledge accumulated so far, this phenomenon cannot be interpreted as a mere consequence of erythrocyte aging. Rather, the binding of oxidized hemoglobin derivatives to plasma membrane plays an important role in determining RBCs senesence (see also Giardina et al., 1995), interacting either with phospolipids or peripheral and integral proteins of the erythrocyte membrane.

In the first place, erythrocyte membranes contain a variety of proteins which may crosslink with hemoglobin (Table 4): 1) crosslinking between spectrin and oxidized Hb is known to cause oxidant hypersensitivity and increased rigidity of the cells (Sauberman et al., 1983; Snyder et al., 1983; Fortier et al., 1988); 2) oxidatively induced methemoglobin-cytoskeletal protein crosslinking is presumably responsible for the increased intracellular concentration of free ionic calcium which occurs during erythrocyte aging in vivo (Aiken et al., 1995); 3) while hemoglobin and met-hemoglobin have a stabilizing effect on the membrane, iHCR may cause an opposite effect by weakening the spectrin-protein 4.1-actin interaction (Jarolim et al., 1990). Such processes may cause a loss in the elastic properties of the cell; this, in turn, leads erythrocytes to navigate less easily the spleen slits and be more frequently phagocytosed.

Second, it is known that binding of hemichrome with a high affinity site in the citoplasmic domain of band 3 (Shaklai et al., 1977a,b) can promote clustering of this integral membrane protein that leads to the exposition of a "senescence antigen" on the outer surface of erythrocyte membrane; subsequent autologous antibody binding and complement fixation result in the rapid removal of the cell by the macrophages (Schluter and Drenckhahn, 1986; Low, 1991; Turrini et al., 1993; Corbett and Golan, 1993).

Third, erythrocyte membranes contain phospholipids rich in polyunsaturated fatty acids that are especially vulnerable to oxidative injury. Abnormal association of Hb oxidation products with membrane proteins may thus target oxidative damage to the

TABLE 4. Principal proteins in human erythrocyte membranes[1]

	Subunit M_r (daltons)	Probable assembly state	Copies per cell
Peripheral proteins			
Spectrin	α = 260,000 β = 225,000	$(\alpha, \beta)_2$ tetramer	10^6
Ankyrin	215,000	Monomer	10^5
Band 4.1	78,000	—	2×10^6
Band 4.2	72,000	—	2×10^6
Band 4.9	45,000	—	5×10^4
Actin	43,000	Oligomer of 12–17 subunits	5×10^5
Glyceraldehyde 3-phosphodehydrogenase	35,000	Tetramer	5×10^5
Band 7	29,000	—	5×10^5
Band 8	23,000	—	10^5
Tropomyosin	29,000	Dimer	7×10^4 dimers
Integral proteins			
Band 3	89,000	Dimer/tetramer	10^6
Glycophorin A	31,000	Dimer	4×10^5
Glycophorin B	23,000	—	$\sim 10^5$
Glycophorin C	29,000	—	$\sim 10^5$

[1] From Bennett, 1985.

lipid component of the membrane (Hebbel, 1986).

In sum, when shifting towards higher oxidation states, hemoglobin begins to crosslink or bind irreversibly with the integral and peripheral proteins, with a noteworthy effect also on lipids (Fig. 1). Therefore, hemoglobin oxidation and binding to membrane components may trigger mechanisms that modify elastic, homeostatic and antigenic properties of the cell. The overall process plays an important role in the clearance of aged erythrocytes. Although Hb-mediated erythrocyte senescence implies different pathways, the weight of evidence points to the dominant role of the immune system's recognition of the "senescence antigen" which follows band 3 clustering (Schluter and Drenckhahn, 1986; Low, 1991; Turrini et al., 1993; Corbett and Golan, 1993).

Oxidative stress is the driving force in this process. Highly reactive species, like O_2^- and H_2O_2, may mediate the gradual oxidation of Hb to irreversible hemichrome-containing Heinz bodies, through the formation of reversible hemichrome and loss of heme moiety (Hebbel et al., 1988). Furthermore, oxygen reactive species and iron decompartmentalization are generated throughout this process and are capable of participating in Hb oxidation and/or promoting oxidative damage of membrane constituents (Misra and Fridovich, 1972; Hebbel, 1986).

There is an increased oxidation and binding to the membrane by hemoglobin in some malaria-linked genetic traits

It is a well-established notion that there is an increased oxidation and binding to the membrane by hemoglobin in erythrocytes containing HbS, HbE and high concentration of HbF, as well as in α- and β-thalassemic and G6PD deficient red cells (Klipstein and Ranney, 1960; Fischer et al., 1975; Shaklai et al., 1981; Giardina et al., 1990).

In comparison with HbA, HbS and HbF (which remains at fairly high concentrations in HbAS cells during the first years of age) have a higher rate of autoxidation, with production of superoxide radicals and heme decompartimentalization (Hebbel et al., 1988). HbS has a higher affinity to the red cell membrane than HbA (Ranney et al., 1993), whereas in HbF and HbE there is a remarkably increased capacity of inducing lipid peroxidation (van den Berg, 1994). Moreover, it has been observed that H_2O_2 treatment of HbF containing erythrocytes results in a marked Hb oxidation, increased crosslinking of Hb to membrane and enhancement of the adsorption of Hb to membrane (Sharma and Premachandra, 1991; Fortier et al., 1988).

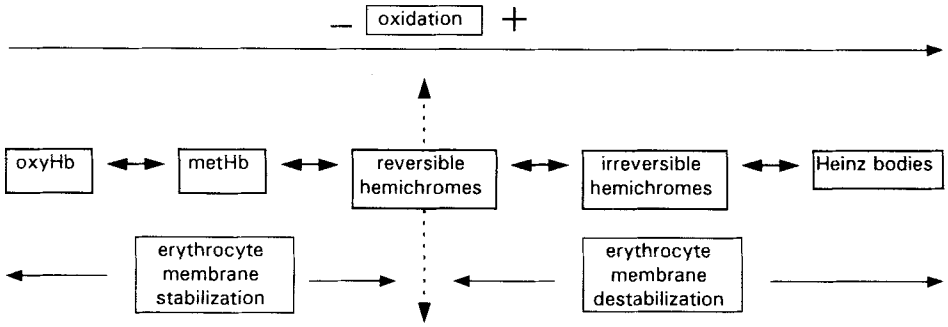

Fig. 1. Schematic representation of the relationship between stability of the erythrocyte membrane and Hb oxidative status. The erythrocyte membrane is stabilized by oxyhemoglobin (oxyHb) and methemoglobin (metHb) and, conversely, destabilized by irreversible hemichromes and Heinz bodies (redrawn from Jarolim et al., 1990).

HbE is particularly susceptible to precipitation when exposed to oxidants (Frischer and Bowman, 1975).

Being inefficient in generating NADPH, G6PD deficient erythrocytes become depleted of GSH when exposed to oxidants. GSH is the substrate of the glutathione peroxidase which shares with catalase the task of detoxifying the erythrocyte by removing hydrogen peroxide. NADPH depletion and impaired GPX activity facilitate oxidation of hemoglobin to methemoglobin and Heinz body formation (Beutler, 1983; Eckman and Eaton, 1979; Scott et al., 1991).

In thalassemic cells the etiology of increased Hb oxidation and binding to the membrane is more complex. A first factor is the high concentration of HbF (in both α- and β-thalassemic cells). Furthermore, in β-thalassemic cells there is an elevated level of HbA_2 which is known to autoxidize slightly faster than HbA and to bind to the red cell membrane, particularly to band 3, to a greater extent either than HbA or hemichrome A (Klipstein and Ranney, 1960; Ranney et al., 1993). Finally, association of partly oxidized globin chains with the cytoskeleton may result in oxidation of adjacent skeletal proteins (protein 4.1 in β-thalassemia and β-spectrin in α-thalassemia), with significant decrease in erythrocyte deformability, and increased generation of met-Hb (Advani et al., 1992; Scott et al., 1990, 1993).

The evidence reviewed clearly points out that there is an increased tendency towards the irreversible binding of oxidized hemoglobin to the membrane. Membrane damage follows substantially the same pathways as in normal cells. In fact, distortion and decreased deformability of the cells may lead to trapping in the microcirculation (Fortier et al., 1988), though membrane lipid damage caused by an excess of oxidants released from abnormal hemoglobins is also an important factor. Once again, a prime factor could be hemichrome binding to the cytoplasmic domain of band 3, leading to clustering of band 3 in the membrane and aggregation of the hemichrome as Heinz bodies (Schluter and Drenckhahn, 1986). This could result in immunologic recognition of the redistributed band 3 by autologous senescent antibodies, leading to removal of the cells from circulation. In addition, generation of Heinz bodies, rarely seen in normal erythrocytes, is facilitated in some mutant erythrocytes, and hemolytic crises are often precipitated by the administration of redox-active drugs, whose ultimate effect is the production of H_2O_2 (Goldberg et al., 1979).

In sum, the same Hb/membrane-induced mechanisms that play an important role in the achievement of normal red cell senescence in 120 days are enhanced in erythrocytes containing HbS, HbE and high concentrations of HbF, as well as in α- and β-thalassemic and G6PD deficient red cells. Hb/membrane interaction plays an im-

portant role in the reduced lifespan and pathological state of erythrocytes carrying the more severe syndromes (like in Hbβ*S homozygotes or carriers of β-major thalassemia) (Weinstein et al., 1954; Bratteyby et al., 1968). Conversely, the advantaged forms in malarial areas (heterozygotes for Hbβ*S, α- and β-thalassemia and in carriers of HbE, high levels of HbF, and G6PD deficiency) are sheltered by an upregulation of antioxidant systems that render erythrocytes mostly asymptomatic (Beutler, 1977; Gerli et al., 1980; Ponzetto-Zimmermann and Natta, 1981; Beretta et al., 1983; Ponnazhagan and Sarkar, 1992). However, they are prone to significant membrane damage in presence of factors which can significantly enhance the oxidation status of Hb, and consequently, its irreversible binding to the membrane components.

Plasmodium falciparum infection may increase membrane bound hemoglobin

During hemoglobin ingestion, the *falciparum* parasite generates H_2O_2 (Gluzman et al., 1992) which is able to egress from the parasite's food vacuole to the host cell compartment (Atamna and Ginsburg, 1993). Another factor which is linked to Hb oxidation is pyrexia, since even a small elevation in temperature can cause a marked increase in the met-Hb to hemichrome conversion rate (Winterbourn, 1990). In *P. falciparum*–infected erythrocytes it would be therefore reasonable to expect an increased Hb oxidation and binding to the membrane.

However, the question of whether *P. falciparum* infection results in an increased oxidative stress for the erythrocyte is rather controversial. Some experimental evidence support the "oxidative" view: 1) in whole blood erythrocyte, spontaneous generation of radical oxygen species was elevated sevenfold in comparison with uninfected controls (Latscha et al., 1987), resulting, at least in part, from infected erythrocytes; 2) lipid peroxidation increases in infected erythrocytes (Wozencraft, 1986); 3) the antimalarial effect of oxidants and oxidant-generating drugs has been assumed to be an indirect demonstration that genetic protective traits function through sensitizing the host cell to oxidants (Vennerstrom and Eaton, 1988). However, it has been pointed out that direct evidence does not provide sufficiently unequivocal data to conclude that *Plasmodium* infection results in an increased oxidative stress within the erythrocyte. In fact, studies on antioxidant molecules (i.e., energy status of parasite/erythrocyte unit, NADH and NADPH, glutathione metabolism, vitamin E, SOD and catalase activities, met-hemoglobin and met-Hb reductase) in infected red cells have showed that the reducing potential tends to increase in the erythrocyte/parasite unit (Hunt and Stocker, 1990). Plasmodial metabolic activities, like that of glutamate dehydrogenase (Roth et al., 1982) which can regenerate antioxidant substances (NADPH) and an upregulation of the hexose monophosphate shunt activity (Atamna et al., 1994), may explain this apparently paradoxical situation. In any case, while it emerges that some cytosolic components may effectively protect the erythrocyte/parasite unit against oxidative stress (Simoes et al., 1992), the current status of knowledge does not address the question of whether *falciparum* infection may contribute to a further and significant increase of membrane-bound hemoglobin.

To give a preliminary answer to this question, we have recently carried out a study on the effect of *falciparum* infection on membrane-bound hemoglobin and some erythrocyte indicators of the intracellular reductive capacity (Destro-Bisol et al., 1993; Giardina et al., 1994). We observed a slight increase in reduced glutathione and ATP content and glutathione peroxidase activity in parasitized erythrocytes (Fig. 2), probably reflecting an upregulation of anti-oxidant systems in response to the oxidative stress promoted by the parasite (Hunt and Stocker, 1990). To obtain more specific information on the consequences of parasite infection at the membrane level, we have detected membrane bound hemoglobin by spectrophotometric analysis or erythrocyte ghosts obtained after repeated washing with a phosphate-buffered solution (Giardina et al., 1990, 1991) which eliminates hemoglobin reversibly bound to the membrane. In comparison with control samples, membrane bound hemoglobin (MBHb) was noticeably increased in plasmodium-infected erythrocytes. Since the para-

sitophorous vacuole membrane is devoid of hemoglobin (Dluzewski et al., 1989a,b), it is reasonable to assert that MBHb in the parasite/erythrocyte unit is bound to the plasma membrane of the host cell. Although we were not able to discriminate between the hemoglobin of the host cell and that released by parasite catabolism (hemozoin), the results suggest that *falciparum* infection may promote binding of oxidized hemoglobins to the erythrocyte membrane. Such results must be taken with caution because of the small sample examined. Furthermore, although erythrocytes obtained from blood venipuncture offer a more realistic picture of the in vivo situation, the interpretation of the results may be confounded by other unpredictable factors. Nonetheless, it is to be stressed that in light of our preliminary results, some previously described properties of infected erythrocyte ghosts may be seen as an effect of the action of membrane bound hemoglobin: 1) the markedly restricted mobility of the transmembrane domain of band 3 mobility (Tilley et al., 1990) following crosslinking via hemichrome (McPherson et al., 1992): 2) the higher susceptibility to oxidative stress of plasma membrane from parasitized erythrocytes depleted of their cytosol (Simoes et al., 1992).

INTERACTION BETWEEN HEMOGLOBIN AND THE ERYTHROCYTE MEMBRANE AS THE PRIMUM MOVENS IN DIFFERENT *FALCIPARUM* MALARIA-LINKED GENETIC TRAITS

On the basis of the evidence reviewed so far, the interaction between oxidized hemoglobins and components of the erythrocyte membranes comply with most of the requirements we have previously formulated. In fact, not only is Hb/membrane interaction an important cellular mechanism for the erythrocytes carrying HbS, HbE, HbF, α- and β-thalassemia, and G6PD deficiency, but it is also reasonable to assume that *falciparum* malaria infection significantly enhances the hemoglobin's tendency towards its irreversible binding to the membrane.

It remains to be seen whether or not Hb/membrane interaction may effectively interfere in the relationship between erythrocytes and *P. falciparum* to a such extent to be actually protective for the erythrocyte. In order to demonstrate that this last requirement may be satisfied, we will show how different in vitro phenomena which are claimed to mediate protection against the *falciparum* parasite in vivo may be seen as the final result of the processes initialized by the Hb/membrane interaction.

Reduced invasion of HbAS and G6PD deficient erythrocytes

Parasite invasion implies the formation of a protein-free patch on the erythrocyte membrane at the site of the invagination. Integral membrane and cytoskeletal proteins are moved away from the site of initial contact along the edges of the junction between the parasite and the erythrocyte (Bannister and Dluzewski, 1990). Tilley et al. (1991) proposed that Melanesian ovalocytes are protected against *falciparum* invasion by a decreased mobility of band 3 protein and increased membrane rigidity that act as a physical barrier to parasite entry. If one considers that the interaction of oxidized hemoglobin with the erythrocyte membrane may promote restricted mobility of band 3 protein and increased membrane rigidity, this pathway could be an important factor for determining the reduced *falciparum* invasion observed in mature erythrocytes (Mons, 1990). Furthermore, the same mechanism could contribute to the reduced in vitro invasion of G6PD deficient erythrocytes exposed to hydrogen peroxide (Kamchonwongpaisan et al., 1989) and HbAS cells cultured at low oxygen tension (Pasvol et al., 1978). Such conclusions are based on the following evidence: 1) it has been shown that when erythrocytes are challenged with H_2O_2, this molecule crosses the membrane and reacts rapidly with Hb, generating very reactive oxidative species, which have Hb, not the membrane, as prime target (van den Berg et al., 1992); 2) deoxygenated Hb shows a higher affinity for band 3 with respect to the oxygenated Hb form (Salhany and Cassoly, 1989).

Death of the parasite within HbAS cells

Following the classical works by Luzzatto et al. (1970) and Roth et al. (1978), intracel-

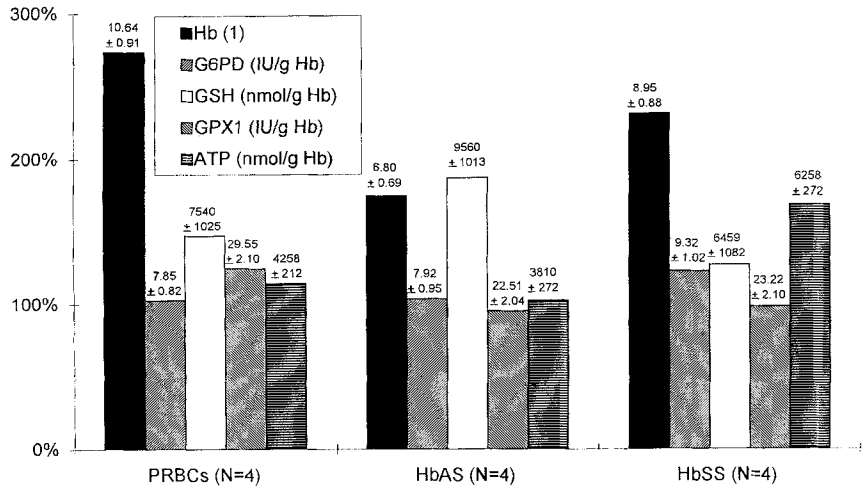

Fig. 2. Determination of GSH and ATP intracellular concentration, GPX1 and G6PD activity, and membrane bound hemoglobin. *Plasmodium falciparum* infected red cells (PRBC) samples contain HbA. HbAS samples are free of *Plasmodium falciparum*. This figure indicates the increased absorbance (expressed in percentage) in the Soret region due to the presence of membrane bound Hb derivatives as compared with the absorbance of Hb-free white ghost profile (for more detail see Giardina et al., 1990).

lular *falciparum* death in HbAS cells is often related to the increased sickling rate induced by the parasite itself (suicidal infection). However, as marked in vivo sickling of HbAS cells is an unlikely event at physiological conditions, the clinical protective effect of such phenomena remains unclear (Yuthavong and Wilairat, 1993). Furthermore, even in cell culture, inhibition of parasite invasion and growth may be independent of sickling (Pasvol et al., 1978).

On the other hand, some of those *falciparum* killing mechanisms which should follow sickling may also be promoted by the Hb/membrane interaction. It has been suggested that sickling may facilitate cellular dehydration and K^+ loss, therefore aiding the formation of the HbS polymer (Friedman et al., 1979). The latter compound is not only a poor substrate for the proteases of the parasite, but it may also interfere with some critical functions of the parasite (Friedman, 1979). Interestingly, sickle cell dehydration and cellular loss of K^+ may also result from the combined effect of oxidative damage and deformation of the red cell membrane (Hebbel, 1991; Brugnara, 1993).

Finally, previous studies gave particular attention to the fact that low oxygen tension may facilitate in vivo sicling of HbAS cells. In fact, *P. falciparum* is the only human specific plasmodium which is confined, for at least part of its erythrocytic cycle, to deep tissues, where the oxygen tension is low. However, it is worth mentioning that such a condition may also enhance Hb binding to the membrane since deoxygenated Hb shows a higher affinity for band 3 with respect to the oxygenated Hb form (Salhany and Cassoly, 1989). Furthermore, since deoxygenated myoglobin is much more prone to oxidation than the corresponding oxygenated forms (Yusa and Shikama, 1987), it could be of interest to check if this property is also valid for hemoglobin.

Phagocytosis of the infected host cell

Hb/membrane interaction might also play an important role in this process by two distinct pathways which have been previously described: 1) binding of hemichromes to band 3 may initialize a series of events which culminate in the rapid removal of the cell by the macrophages; 2) elastic properties of erythrocyte membranes may be seriously compromised following crosslinking of peripheral proteins with hemoglobin derivatives, facilitating phagocytosis of the eryth-

rocytes during their navigation of the spleen slits.

Protection afforded by dietary intakes of oxidative substances

Consumption of fava beans is thought to be necessary to activate protection against the parasite in HbAE and G6PD Mediterranean deficient cells (Kitayaporn et al., 1992; Huheey and Martin, 1975). There is some evidence that Hb/membrane interaction may play a major role in this case too.

Vicia faba contains β-glucosides (vicine and convicine) which are hydrolyzed in the gastrointestinal tract to form two unstable pyrimidine aglycones, divicine and isouramil (Chevion et al., 1982). Divicine is capable of inhibiting parasite development in cultured G6PD Mediterranean deficient erythrocytes, whereas isouramil has a strong antimalarial effect in G6PD normal mice, infected with *P. vinckei.* Both these compounds generate hydrogen peroxide and free radical species (Chevion et al., 1982), which presumably have hemoglobin as a main target (van den Berg et al., 1992). Finally, in vitro studies by Mavelli et al. (1984, 1985) further support the involvement of Hb/membrane interaction. In this work, it was observed that membrane damage in G6PD Mediterranean deficient erythrocytes exposed to divicine implies a significant inactivation of glutathione peroxidase, an enzyme which detoxifies the erythrocyte by removing H_2O_2, acting preferentially at membrane level (Bozzi et al., 1976); the authors noted that membrane damage may be blocked if Hb oxidation is prevented and, conversely, remarkably enhanced if the protein is transformed into met-Hb.

A COHERENT FRAMEWORK

In Figure 3 we have tried to synthesize the various pathways by which the interaction between oxidized hemoglobin and the erythrocyte membrane may counteract the proliferation of *P. falciparum.* Reduced invasion (in HbAS and G6PD deficient subjects) and intracellular *falciparum* death (HbAS) probably provide a first line of defense which facilitates subsequent protective pathways reducing parasitemia. Phagocytosis of infected erythrocytes is expected to be the most

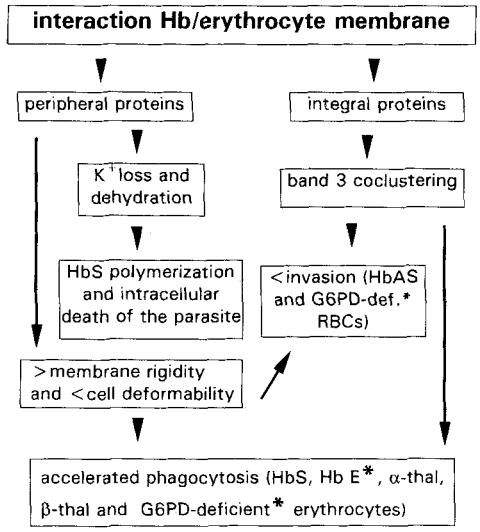

Fig. 3. Schematic summary of the pathways by which the interaction between oxidized hemoglobin and the erythrocyte membrane may end up by counteracting the proliferation of *Plasmodium falciparum* (see text for discussion). The asterisk indicates that protection against the *falciparum* parasite in carriers of the common Mediterranean form of G6PD deficiency and of HbE probably requires the dietary intake of H_2O_2 generating-compounds.

important pathway promoted by the Hb/membrane interaction. An efficient erythrophagocytosis of ring forms may, in fact, remove significant amounts of erythrocytes from circulation, avoiding the impairment of the macrophage function by hemozoin. By decreasing the number of infected erythrocytes cells that may adhere to the venular vascular endothelium of internal organs, accelerated phagocytosis may significantly reduce the risk of developing those microcirculatory obstructions which cause cerebral malaria. Given the predominant role of erythrophagocytosis, our model implies that protection against *P. falciparum* is substantially achieved by accelerating the same mechanisms responsible for normal erythrocyte senescence. In this last process a pivotal role is probably played by the interaction between hemoglobin, the most abundant protein in the cytoplasm, and band 3, the most prominent protein in the membrane, which cooperate to transduce information regarding the infection/senescence status of the cell to the reticuloendothelial system.

It may be noted that in those conditions where compensatory enzyme activity by the parasite is present (G6PD deficiency), or the instability of hemoglobin is mild (HbE), specific dietary intakes may help provide protection, by increasing Hb oxidation and membrane binding. Another interesting observation is that HbS is capable of activating pathways that influence both invasion and intracellular survival of the parasite and clearance of the erythrocyte/parasite unit. Correspondingly, HbS is also believed to be the most effective in protecting its carriers against *falciparum* malaria. Also in the case of HbS, accelerated phagocytosis of infected erythrocytes would provide the most efficient level of protection. It has been in fact observed that splenectomy of β*S transgenic mice completely reverses the protection against *Plasmodium chabaudi adami* infection (Shear 1993; Shear et al., 1994). Furthermore, HbSS subjects, who have anatomical and functional asplenia, have no or reduced resistance against the infection (Luzzatto and Pinching, 1990).

Our model does not deny the possibility that other pathways, such as the decreased rosetting ability of mycrocytic thalassemic erythrocytes (Carlson et al., 1993, 1994) or the inhibitory role on *falciparum* protein synthesis by oxidized glutathione which accumulates in the parasitized G6PD deficient erythrocytes (Kosower and Kosower, 1970), interact in determining the overall antimalarial capacity of mutant erythrocytes. On the other hand, whereas the malaria-linked genetic traits and biochemical properties of HbS have been deeply investigated, less is known about other malaria-linked traits like HbE and HbF. It would therefore be interesting to see whether additional protective pathways might be promoted by the Hb/membrane interaction.

TWO CASE STUDIES

The effect of cyanogens on the interaction between oxidized hemoglobins and membrane may help explain the relationship between cassava consumption and HbS distribution in Liberia

Jackson (1990) found evidence that the regular consumption of cassava (*Manihot esculenta*) by some human groups may provide protection against *P. falciparum* and influence Hbβ*S gene frequencies. Jackson's survey was carried out in Liberia, where malaria is holoendemic and cassava has been an important dietary component since 1600. The author focuses her attention on the cassava-derived cyanogen compounds (CN^-, SCN^- and CNO^-), known to have an inhibitory effect on sickling and intraerythrocytic growth of the *falciparum* parasite. Jackson observed that populations from northwestern and western areas of the country are characterized by lower dietary intakes of cyanogens, lower mean *Plasmodium*-positive antibody titers, higher mean prevalence of clinical *falciparum* malaria and higher Hbβ*S frequencies (11%). Conversely, in populations from southeastern and central Liberia she found that higher dietary cyanogen intakes are associated with lower Hbβ*S frequencies (2%), higher mean *Plasmodium*-positive antibody titers and lower mean prevalence of clinical *falciparum* malaria. On the basis of these findings, the author proposed two distinct patterns of interaction between Hb and cyanogens: 1) in the lower dietary intakes, typical of northwestern and western regions, cyanogens may interact with HbS and inhibit sickling, slightly reducing the biological fitness of heterozygotes for the Hbβ*S allele and improving that of homozygous Hbβ*S individuals; 2) in higher dietary intakes, cyanogen itself may inhibit parasite growth by interacting with important proteins of *Plasmodium falciparum*. In the first case, the slight improvement of HbSS fitness is expected to favor an increase of Hbβ*S frequency over time; in the second case, since the diet is thought to be capable of markedly reducing the clinical severity of *falciparum* malaria, the selective pressure which maintains Hbβ*S frequency would be reduced to such an extent that the frequency of the mutant gene is expected to decrease over time.

There is evidence suggesting that the interaction between oxidized hemoglobin and the erythrocyte membrane may be relevant to the Jackson's hypothesis. In an in vitro study, Giardina et al. (1991) observed that Hb binding to the erythrocyte membrane may be remarkably decreased (by about 50%) by addition of solutions containing

CN^-. Such a result may have two main implications. From a general point of view, CN^- being a ligand of hemoglobin's ferric form, the release of Hb from the membrane suggests that almost 50% of membrane-bound Hb is made up by met-Hb and/or the various oxidation intermediates of the molecule's progressive oxidation. Relating this study to the Jackson's hypothesis, if we assume that this in vitro interaction is also relevant to in vivo phenomena, it can be hypothesized that high dietary intakes of CN^- may interfere with the processes initialized by the Hb/membrane interaction. The cyanogens could, therefore, impair the biochemical process at the basis of Hbβ*S protective capacity. As an ultimate consequence, we expect a marked reduction of the selective advantage of Hbβ*S in malarial areas in those populations with high cyanogen dietary intakes and, consequently, a progressive decline of the frequency of the Hbβ*S allele. Obviously, our standpoint is not mutually exclusive of that of Jackson (1990), since the effect of the biochemical mechanism we suggest may potentially cumulate with the hypothesized cyanogen capacity in undermining the environmental conditions behind the Hbβ*S selective advantage (Jackson, 1990).

Can the GPX1*2 allele product impair the protection against *falciparum* malaria afforded by HbS?

The erythrocyte enzyme glutathione peroxidase (GPX1) displays a genetic polymorphism among populations inhabiting (or coming from) areas with malaria endemicity (Destro-Bisol and Spedini, 1989). The enzyme encoded by the allele GPX1*2 is characterized by a higher peroxidase activity with respect to the GPX1*1 allele product, so that the GPX1 phenotype 2-1 has, on an average, about 1.5 times the catalytic activity observed in GPX1 phenotype 1 (Meera Khan et al., 1986). It has been proposed that the higher peroxidase activity of GPX1*2 could be detrimental to *P. falciparum* by lowering the intracellular availability of GSH, a compound essential for parasite growth and development (Meera Khan et al., 1986). However, from our point of view a marked increase of GPX1 activity could end up by impairing the protection afforded by HbS against the *falciparum* parasite. In fact, it has been shown that an upregulation of GPX1 activity may counteract the enhanced Hb oxidation in HbAS (Beretta et al., 1983) and G6PD deficient subjects (Beutler et al., 1977) and in α- and β-thalassemia heterozygotes (Gerli et al., 1980; Ponzetto-Zimmermann and Natta, 1981; Ponnazhagan and Sarkar, 1992) by removing H_2O_2 which escapes catalase action and diffuses to the membrane (Bozzi et al., 1976). It may be therefore hypothesized that in GPX1*2 carriers, the remarkable elevation of peroxidase capacity might compensate Hb oxidation resulting from the combined effect of HbS and *P. falciparum* invasion. As a consequence, those HbAS individuals who also carry the GPX1*2 allele could no longer be protected against the parasite, and would have a decreased chance of passing the first 5 years of life in which immune-mediated resistance is not yet effective. On the contrary, no significant consequences for survival are expected when GPX1*2 is carried by an HbAA individual. Therefore, our hypothesis predicts that GPX1*2 frequency will be lower among HbAS than HbAA individuals. In a previous paper, we reported on the distribution at the GPX1 and Hbβ loci in some populations from Congo, Cameroon and Benin (Destro-Bisol and Spedini, 1989). With only one exception (South Bakaka), in all the groups the GPX1*2 frequency was lower in the HbAS than in the corresponding HbAA subsample, and the difference obtained after pooling the population samples was statistically significant ($P = 0.032$).[1] The analysis has been now extended to other populations of the same geographical area (about 3,500 individuals), and again we observed a systematic trend towards a negative association between the Hbβ*S and GPX1*2 alleles (Destro-Bisol et al., in preparation). Furthermore, we have found that this behavior is specific to the Hb/GPX1 pair ($P < 0.036$), whereas the remaining pairs of loci we have considered (Hb, Esd, CAII, GPX1) showed no significant departure from the expectations of a random association (Destro-Bisol et al., 1995).

[1]This value is different from that reported in Destrol-Bisol and Spedini (1989) because it was obtained by including the South Bakakas in the pooled sample.

CONCLUDING REMARKS

The main conclusion of this paper is that the interaction between oxidized hemoglobin and the erythrocyte membrane is not only an important contributor of erythrocyte senescence, but it also may be the primum movens of various cellular mechanisms which act against the erythrocytic proliferation of *P. falciparum.* This new perspective proved also useful in a more strictly anthropological context, providing valuable explanations for the influence of dietary and genetic factors on the distribution of the HbS allele in various sub-Saharan populations.

Although our view is based on a large body of evidence, its final validation still needs further confirmatory results. To achieve this last goal, it is important that the experimental design of future research on malaria-linked genetic traits will systematically take into account the possible role of mechanisms promoted by the interaction between oxidized hemoglobin and the erythrocyte membrane.

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LITERATURE CITED

Advani R, Sorenson S, Shinar E, Lande W, Rachmilewitz E, and Schrier SL (1992) Characterization and comparison of the red blood cell membrane damage in severe human alpha- and beta-thalassemia. Blood. *79:*1058–1063.

Aiken NR, Galey WR, and Satterlee JD (1995). A peroxidative model of human erythrocyte intracellular Ca^{2+} changes with in vivo cell aging: Measurement by 19F-NMR spectroscopy. Biochim. Biophys. Acta. *1270:* 52–57.

Allison AC (1954a) Protection afforded by sickle cell trait against subtertian malaria infection. Br. Med. J. *1:*1346–1349.

Allison AC (1954b) The distribution of the sickle cell trait in East Africa and elsewhere, and its apparent relationship to the incidence of subtertian malaria. Trans. R. Soc. Trop. Med. Hyg. *48:*312–318.

Allison AC (1954c) Note on sickle-cell polymorphism. Ann. Hum. Genet. *19:*39–57.

Allison AC (1960) Glucose 6 phosphate dehydrogenase deficiency in red blood cells of East Africans. Nature *186:*531–532.

Allison AC, and Euaui EM (1983) The role of cell-mediated immunity in resistance to oxidant stress. Immunol. *1:*361–392.

Ammermann AJ, and Cavalli-Sforza LL (1984) The Neolithic transition and the genetics of populations in Europe. Princeton, N.J.: Princeton University Press.

Anderson BB, Giuberti M, Perry GM, Salsini G, Casadio I, and Vullo C (1993) Low red blood cell glutathione reductase and pyridoxine phosphate oxidase activities not related to dietary riboflavin: Selection by malaria? Am. J. Clin. Nutr. *57:*666–672.

Angel L (1966) Porotic hyperostosis, anemias, malarias and marshes in the prehistoric eastern Mediterranean. Science *153:*760–763.

Arese P, Turrini F, and Ginsburg H (1991) Erythrophagocytosis in malaria: Host defence or menace to the macrophage? Parasitol. Today *7:*25–28.

Arese P, Cappadoro M, and Giribaldi e Turrini F (1994) The malaria/G6PD hypothesis revisited: Reply. Parasitol. Today *10:*262–263.

Ascenzi A (1979) A problem in paleopathology: The origin of thalassemia in Italy. Virchows Arch. *384:* 121–130.

Atamna H, and Ginsburg H (1993) Origin of reactive oxygen species in erythrocytes infected with *Pasmodium falciparum.* Mol. Biochem. Parasitol. *61:*231–241.

Atamna H, Pascarmona G, and Ginsburg H (1994) Hexose-monophosphate shunt activity in intact *Plasmodium falciparum*–infected erythrocytes and in free parasites. Mol. Biochem. Parasitol. *67:*79–89.

Baer A, Lie-Injo LE, Welch QB, and Lewis AN (1976) Genetic factors and malaria in the Temuan. Am. J. Hum. Genet. *28:*179–188.

Bannister LH, and Dluzewski AR (1990) The ultrastructure of red cell invasion in malaria infections: A review. Blood Cells *16:*257–292.

Beet EA (1946) Sickle cell disease in the Balovale district of North Rhodesia. East Afr. Med. J. *23:*75.

Beet EA (1947) Sickle cell disease in the Balovale district of Northern Rhodesia. East Afr. Med. J. *24:*212–222.

Bennett V (1985) The membrane skeleton of human erythrocytes and its implications for more complex cells. Ann. Rev. Biochem. *54:*273–304.

Beretta L, Gerli GC, Ferraresi R, Agostoni A, Gualandri V, and Orsini GB (1983) Antioxidant system in sickle red cells. Acta Haematol. *70:*194–197.

Beutler E (1977) Glucose-6-phosphate dehydrogenase

deficiency and red cell glutathione peroxidase. Blood *49*:467–469.

Beutler E (1983) Glucose-6-Phosphate dehydrogenase deficiency. In JB Stanbury, J Wyngaarden, DS Fredickson, and J Goldstein (eds.): The metabolic basis of inherited Disease. New York: McGraw Hill, pp. 1629–1653.

Bowman JE, and Murray RF (1990) Genetic Variation and Disorders in Peoples of African Origin. Baltimore: John Hopkins University Press, pp. 229–243.

Boyd MF (1949) Malariology. Philadelphia: W.B. Saunders, pp. 3–25.

Bozzi A, Mavelli I, Finazzi Agrò A, Strom R, Wolf AM, Mondovì B, and Rotilio G (1976) Enzyme defense against reactive oxygen derivatives. II. Erythrocytes and tumor cells. Mol. Cell. Biochem. *10*:11–16.

Bratteyby LE, Garby L, Groth TC, Schneider W, and Wadman M (1968) Studies on erythrokinetics in infancy. XIII. The mean life span and the life span frequency of red blood cells formed during fetal life. Acta Pediatr. Scand. *57*:311–320.

Bray RS (1958) Studies on malaria in chimpanzees. VI. *Laverania falciparum*. Am. J. Trop. Med. Hyg. *7*:20–24.

Brockelman CR, Wongsattayanont B, Tan-Arya P, and Fucharoen S (1987) Thalassemic erythrocytes inhibit in vitro growth of *Plasmodium falciparum*. J. Clin. Microb. *25*:56–60.

Brown PJ (1981) New considerations on the distribution of malaria, thalassemia and glucose-6-phosphate dehydrogenase deficiency in Sardinia. Hum. Biol. *53*:367–382.

Bruce-Chwatt LJ (1965) Paleogenesis and paleoepidemiology of primate malaria. Bull. World Health Organ. *32*:363–387.

Bruce-Chwatt LJ (1988) History of malaria from prehistory to eradication. In WL Wernsdorfer and I McGregor (eds.): Malaria: Principles and Practice of Malariology, vol. 1. Edinburgh: Churchill Livingstone, pp. 1–59.

Brugnara C (1993) Membrane transport of Na and K and cell dehydration in sickle erythrocytes. Experientia *49*:100–109.

Bunyaratvey A, Butthep P, and Yuthavong Y (1986) Increased phagocytois of *Plasmodium falciparum* infected erythrocytes with HbE by peripheral blood monocytes. Acta Haematol. *76*:155–158.

Carlson J (1993) Erythrocyte rosetting in *Plasmodium falciparum* malaria, with special reference to the pathogenesis of cerebral malaria. Scand. J. Infect. Dis. Suppl. *86*:1–79.

Carlson J, Nash GB, Gabutti V, al-Yaman F, and Wahlgren M (1994) Natural protection against severe *Plasmodium falciparum* malaria due to impaired rosette formation. Blood *84*:3909–3914.

Carnevale P, Robert V, Molez JF, and Boudon D (1984). Epidémiologie générale: Faciées épidémiologique des paludismes en Afrique subsaharienne. Etudes médicales *3*:123–133.

Cattani J, Gibson DF, Alpers MP, and Crane GG (1987) Hereditary ovalocytosis and reduced susceptibility to malaria in Papua New Guinea. Trans. R. Soc. Trop. Med. Hyg. *81*:705–709.

Cavalli-Sforza LL and Bodmer WF (1971) The Genetics of Human Populations. San Francisco: W. H. Freeman.

Chernoff AI (1959) The distribution of the thalassemia gene: A historical review. Blood *14*:899–905.

Chevion M, Navok T, Glaser G, and Mager J (1982) The chemistry of isouramil and divicine and their reactions with glutathione. Eur. J. Biochem. *127*:405–409.

Chiu D, and Lubin B (1989) Oxidative hemoglobin denaturation and RBC destruction. The effect of heme on red cell membranes. Semin. Haematol. *26*:128–135.

Coatney GR, Collins WE, Warrenn McW, and Contacos PG (1971) The Primate Malarias. Washington, D.C.: U.S. Government Printing Office.

Coluzzi M (1985) Chromosomal inversion intergradation and incipient speciation in *Anopheles gambiae*. Boll. Zool. *52*:45–63.

Coluzzi M (1994) Malaria and the Afrotropical ecosystems: Impact of man-made environmental changes. Parassitologia *36*:223–227.

Corbett JD, and Golan DE (1993) Band 3 and glycophorin are progressively aggregated in density-fractionated sickle and normal red blood cells: Evidence from rotational and lateral mobility studies. J. Clin. Invest. *91*:208–217.

Cox FE (1991) Malaria vaccines, progress and problems. Trends Biotechnol. *9*:389–394.

Das BM, Deka R, and Flatz G (1975) Predominance of the haemoglobin E gene in a Mongoloid population in Assam (India). Humangenetik *30*:187–191.

Descamps-Latscha B, Lunel-Fabianif, Kara-Binis A, and Druime P (1987) Generation of reactive oxygen species in whole blood from patients with acute *Falciparum* malaria. Parasite Immunol. *9*:275–279.

Destro-Bisol G, and Spedini G (1989) Anthropological survey on red cell glutathione peroxidase (GPX1) polymorphism in Central Western Africa: A tentative hypothesis on the interaction between GPX1*2 and Hb beta*S allelic products. Am. J. Phys. Anthropol. *79*:217–224.

Destro-Bisol G, Scatena R, and Giardina B (1993) Stress ossidativo, membrana eritrocitaria e meta-emoglobina e loro possibile ruolo nei meccanismi molecolari di adattamento genetico dell'eritrocita alla malaria. Oral communication at the 10th Congress of the Italian Anthropologists, Pisa, 8–10 September 1993.

Destro-Bisol G, Sansonetti B, Battaggia C, and Spedini G (1995) Nuovi dati sulla distribuzione dei polimorfismi dell'emoglobina (Hbβ) e della glutatione perossidasi eritrocitaria (GPX1) in alcune popolazioni dell'-Africa subsahariana. Oral communication at the 11th Congress of the Italian Anthropologists, Isernia, 8–10 September 1995.

Dluzewski AR, Rangahrachi K, Wilson RJM, and Gratzer WB (1989a) A cytoplasmic requirement of red cell invasion by malarial parasite. Mol. Biochem. Parasitol. *9*:145–160.

Dluzewski AR, Fryer PR, Griffiths S, Wilson RJM, and Gratzer WB (1989b) Red cell membrane protein distribution during malarial invasion. J. Cell Sci. *92*: 691–699.

Dunn FL (1965) On the antiquity of malaria in the western hemisphere. Hum. Biol. *37*:385–393.

Eckman JR, and Eaton WE (1979) Dependence of plas-

modial glutathione metabolism on the host cell. Nature *278*:754–756.

Effertz O (1909) Malaria in tropical America and among Indians. Janus *14*:246–261.

Ehret C (1984) Historical/linguistic evidence for early African food production. In J Desmond Clark and SA Brandt (eds): From Hunters to Gatherers. Berkeley: University of California Press, pp. 26–35.

Etkin N, and Eaton JW (1975) Malaria-induced erythrocyte oxidant sensitivity. In GJ Brewer (ed.): Erythrocyte Structure and Function. New York: Alan Liss, p. 219.

Filon D, Faerman M, Smith P, and Oppenheim A (1995) Sequence analysis reveals a β-thalassemia mutation in the DNA of skeletal remains from the archaeological site of Akhziv, Israel. Nature Genet. *9*:365–368.

Fischer S, Nagel RL, Bookckin RM, Roth EF Jr, and Tellez-Nagel I (1975) The binding of hemoglobin to membranes of normal and sickle erythrocytes. Biochim. Biophys. Acta *375*:422–433.

Flatz G, Pik C, and Sundharagiati B (1964) Malaria and hemoglobin E in Thailand. Lancet *2*:385–387.

Flint J, Harding RM, Clegg JB, and Boyce AJ (1993) Why are some genetic diseases common? Distinguishing selection from other processes by molecular analysis of globine gene variants. Hum. Genet. *91*:91–117.

Foo LC, Rekhraj V, Chiang GL, and Mak JW (1992) Ovalocytosis protects against severe malaria parasitemia in the Malayan aborigines. Am. J. Trop. Med. Hyg. *47*:271–275.

Fortier N, Snyder ML, Garver F, Kiefer C, McKenney J, and Mohandas N (1988) The relationship between in vivo generated hemoglobin skeletal protein complex and increased red cell membrane rigidity. Blood *71*:1427–1431.

Friedman MJ (1978) Erythrocytic mechanism of sickle cell resistance to malaria. Proc. Natl. Acad. Sci. U.S.A. *75*:1994–1997.

Friedman MJ (1979) Oxidant damage mediates variant red cell resistance to malaria. Nature *280*:245–247.

Friedman MJ, Roth EF, Nagel RL, and Trager W (1979) The role of hemoglobins C, S and Nbalt in the inhibition of malaria parasite development in vitro. Am. J. Trop. Med. Hyg. *28*:777–780.

Frischer H, and Bowman J (1975) Hemoglobin E, an oxidatively unstable mutation. J. Lab. Clin. Med. *85*:531–539.

Garnham PCC (1966) Malaria parasites and other haemosporidia. Oxford: Blackwell.

Genton B, Al-Yaman F, Mgone CS, Alexander N, Paniu MM, Alpers MP, and Mokela D (1995) Ovalocytosis and cerebral malaria. Nature *378*:564–565.

Gerli GC, Beretta L, Bianchi M, Pellegatta A, and Agostoni A (1980) Erythrocyte superoxide dismutase, catalase and glutathione peroxidase activities in β-thalassaemia (major and minor). Scand. J. Haematol. *25*:87–92.

Giardina B, Condò SG, Clemente ME, Scatena R, Pellegrini MG, Corda M, Sanna MT, Martorana MC, Aebischer ML, and Angeloni P (1990) Aspetti fisiologici e patologici dell'interazione metemoglobina-membrana eritrocitaria. Tribuna Biologica e Medica *1*:12–14.

Giardina B, Scatena R, Clementi ME, Ramacci MT, Maccari F, Cerroni L, and Condò S (1991) Selective binding of met-hemoglobin to erythrocytic membrane: A possible involvement in red blood cell aging. In M Magnani and A De Flora (eds.): Red Blood Cell Aging. New York: Plenum Press, pp. 75–84.

Giardina B, Destro-Bisol G, Scatena R, Cerroni L, Nocca R, Santini SA, Mordente A, Aebischer ML, Bodo JM, and Spedini G (1994) Hemoglobin mediated oxidative damage to the red cell membrane and its relevance to some erythrocyte genetic adaptations to malaria. Riv. Antropol. *72*:81–92.

Giardina B, Messana I, Scatena R, and Castagnola M (1995) The multiple functions of hemoglobin. Crit. Rev. Biochem. Mol. Biol. *30*:165–196.

Giulivi C, and Davies KJA (1990) A novel antioxidant role for hemoglobin. The comproportionation of ferryl-hemoglobin with oxyhemoglobin. J. Biol. Chem. *226*:19453–19460.

Gluzman IY, Francis SE, Oksman A, Smith CE, Duffin KL, and Goldberg DE (1994) Order and specificity of the *Plasmodium falciparum* hemoglobin degradation pathway. J. Clin. Invest. *93*:1602–1608.

Goldberg B, Stern S, Peisach J, and Blumberg WF (1979) The detection of superoxide anion from the reaction of oxyhemoglobin and phenylhydrazine using EPR spectroscopy. Experientia *35*:488–489.

Goueffon S, and du Saussay C (1969) Enquète systématique sur l'hémoglobine E at la glucose-6-phosphate déhydroggénase au Cambodge (Octobre 1965–Jun 1966). Bull. Soc. Pathol. Exot. *62*:1118–1132.

Greene LS (1993) G6PD deficiency as protection against *falciparum* malaria: An epidemiologic critique of population and experimental data. Yrbk. Phys. Anthropol. *36*:153–178.

Greenwood MB, Bradley AK, Greenwood AM, Byass P, Jammen K, Marsh K, Tulloch S, Oldfield FSJ, and Hayes R (1987) Mortality and morbidity from malaria among children in a rural area of the Gambia, West Africa. Trans. R. Soc. Trop. Med. Hyg. *81*:271–275.

Grmek M (1988) Diseases in the Ancient Greek World. Baltimore and London: Johns Hopkins University Press.

Grmek (1994) La Malaria dans la Méditerranée orientale. Parassitologia *36*:1–6.

Haldane JBS (1949a) Disease and evolution. Ricerca Scientifica *19*:68–76.

Haldane JBS (1949b) The rate of mutation of human genes. Proceedings of the Seventh International Congress of Genetics. Hereditas (suppl.) *35*:267–271.

Haldane JBS (1957) Natural selection in man. Acta gene. stat. Med. *6*:321–332.

Hebbel RP (1986) Erythrocyte antioxidants and membrane vulnerability. J. Lab. Clin. Med. *107*:401–404.

Hebbel RP (1991) Beyond hemoglobin polymerization: the red blood cell membrane and sickle disease pathophysiology. Blood *77*:214–237.

Hebbel RP, Morgan WT, Eaton JW, and Hedlund BE (1988) Accelerated autoxidation and heme loss due to the instability of sickle hemoglobin. Proc. Natl. Acad. Sci. U.S.A. *85*:237–241.

Helck W, and Otto E (eds,) (1982): Lexikon der Aegyptologie. Wiesbaden: O. Harrassowitz, vol. 4, p. 705.

Hill AV (1992) Malaria resistance genes: a natural selection. Trans. R. Soc. Trop. Med. Hyg. *86*:225–226.

Hill AVS, Allsopp CEM, Kwiatkowski D, Anstey NM, Twumasi P, Rowe PA, Bennett S, Brewster D, McMichael AJ, and Greenwood BM (1991) Common west african HLA antigens are associated with protection from severe malaria. Nature *352*:595–600.

Huheey RP, and Martin DL (1975) Malaria, favism and glucose 6 phosphate dehydrogenase deficiency. Experientia *31*:1145–1147.

Hunt NH, and Stocker R (1990) Oxidative stress and the redox status of malaria infected erythrocytes. Blood Cells *16*:499–526.

Jackson FMC (1990) Two evolutionary models for the interactions of dietary organic cyanogens, hemoglobin and *falciparum* malaria. Am. J. Hum. Biol. *2*:521–532.

Jacob F (1977) Evolution and tinkering. Science *196*:1161–1166.

Jarolim P, Lahav M, Liu SC, and Palek J (1990) Effect of hemoglobin oxidation products on the stability of red cell membrane skeletons and the associations of skeletal proteins: Correlation with a release of hemin. Blood *76*:2125–2131.

Kamchonwongpaisan S, Bunyiaratvej A, Wanachiwanawin W, and Yuthavong Y (1989) Susceptibility to hydrogen peroxide of *Plasmodium falciparum* infecting glucose-6-phosphate dehydrogenase-deficient erythrocytes. Parasitology *99*:171–174.

Kidson C, Lamont G, Saul A, and Nurse GT (1981) Ovalocytic erythrocytes are resistant to invasion by malaria parasites in culture. Proc. Natl. Acad. Sci. U.S.A. *78*:5829–5832.

Kitayaporn D, Nelson KE, Cgharoenlarp P, and Pholpthi T (1992) Haemoglobin-E in the presence of oxidative substances from fava bean may be protective against *Plasmodium falciparum* malaria. Trans. R. Soc. Trop. Med. Hyg. *86*:240–244.

Klipstein FA and Ranney HM (1960) Electrophoretic components of the hemoglobin of red cell membranes J. Clin. Invest. *39*:1894–1899.

Knell AJ (1991) Malaria. Oxford: Oxford University Press.

Kosower NS, and Kosower EM (1970) Molecular basis for selective advantage of G6PD deficient individuals exposed to malaria. Lancet *II*:1343–1344.

Livingstone FB (1958) Anthropological implications of sickle cell gene distribution in West Africa. Am. Anthropol. *60*:533–562.

Livingstone FB (1971) Malaria and human polymorphisms. Ann. Rev. Genet. *5*:33–64.

Livingstone FB (1984) The duffy blood groups, vivax malaria and malaria selection in human populations: A review. Hum. Biol. *56*:413–425.

Low PS (1991) Role of hemoglobin denaturation and band 3 clustering in initiating red cell removal. In M Magnani and A De Flora (eds.): Red Blood Cell Aging. New York: Plenum Press, pp. 173–183.

Luzzatto L (1979) Genetics of red cells and susceptibility to malaria. Blood *54*:961–976.

Luzzato L, and Pinching AJ (1990) Innate resistance to malaria: The intraerythrocitic cycle. Blood Cells *16*:340–347.

Luzzatto L, Nwachukes-Jarrett ES, and Reddy S (1970) Increased sickling of parasitized erythrocytes as mechanism of resistance against malaria in the sickle-cell trait. Lancet *1*:319–322.

Marshall LB (1993) Disease ecologies of Australia and Oceania. In KF Kiple (ed.): The Cambridge World History of Human Disease. Cambridge: Cambridge University Press, pp. 482–496.

Martin SK, Miller LH, Kark JA, Hicks CU, Haut MJ, Okoye VC, and Esan GJF (1978) Low erythrocyte pyridoxal-kinase activity in blacks: Its possible relation to *falciparum* malaria. Lancet *1*:466–468.

Mattingly PF (1976) Evolution of the malarias: The problem of origins. Parassitologia *18*:1–18.

Mavelli I, Ciriolo RM, Rossi L, Meloni T, Forteleone G, De Flora A, Benati U, Morelli A, and Rotilio G (1984) Favism: A hemolytic disease associated with increased superoxide dismutase and decreased glutathione peroxidase activities in red blood cells. Eur. J. Biochem. *139*:13–18.

Mavelli I, Ciriolo RM, and Rotilio G (1985) Inactivation of red cell glutathione peroxidase by divicine and its relation to the hemolysis of favism. Biochem. Biophys. Acta *847*:280–284.

McPherson R.A., Sawyer WH, and Tilley L (1992) Rotational diffusion of the erythrocyte integral membrane protein band 3: Effect of hemichrome binding. Biochemistry *31*:512–518.

Meera Khan P, Verma C, Wijnen LMM, Wijnen JTh, Prins HK, and LE Nijenhuis (1986) Electrotypes and formal genetics of red cell glutathione peroxidase (GPX1) in the Djuka of Surinam. Am. J. Hum. Genet. *38*:712–723.

Misra HP, and Fridovich I (1972) The generation of superoxide radical during the autoxidation of hemoglobin. J. Biol. Chem. *21*:6960–6962.

Mons B (1990) Preferential invasion of malarial merozoites into young blood red cells. Blood Cells *16*:299–312.

Motulsky AG (1960) Metabolic polymorphisms and the role of infectious disease in human evolution. Hum. Biol. *32*:28–62.

Motulsky AG (1989) Update to "Metabolic polymorphisms and the role of infectious disease in human evolution." Hum. Biol. *61*:870–877.

Moya CE, Shah H, and Sodeman TM (1985) The erythrocyte. In: Sodeman's Pathologic Physiology, 7th ed. Baltimore: W.B. Saunders.

Murray MJ, Murray AB, Murray NJ, and Murray MB (1978) Diet and cerebral malaria: The effect of famine and refeeding. Am. J. Clin. Nutr. *31*:57–61.

Nagel RL (1990) Innate resistance to malaria: The intraerythrocytic cycle. Blood Cells *16*:321–339.

Nagel RL, Raventos-Suarez C, Fabry HE, and Tanowitz H (1981) Impairment of the growth of *Plasmodium falciparum* in HbEE erythrocytes. J. Clin. Invest. *68*:303–305.

Nurse GT (1979) Iron, the thalassemias and malaria. Lancet *2*:938–940.

Olson IA, and Nagel RL (1986) Synchronized cultures of *Plasmodium falciparum* in abnormal red cells; the mechanism of inhibition of growth in Hb CC cells. Blood *67*:997–1001.

Oppenheimer SJ, Higgs DR, Weatherall DJ, Barker J, and Spark A (1984) Alpha thalassemia in Papua New Guinea. Lancet *1*:424–426.

Pasvol G, Weatherall DJ, and Wilson RJM (1977) Effects of foetal haemoglobin on susceptibility of red cells to *Plasmodium falciparum*. Nature *270*:171.

Pasvol G, Weatherall DJ, and Wilson RJM (1978) Cellular mechanism for the protective effect of haemoglobin S against *P. falciparum* malaria. Nature *274*:701–703.

Pasvol G, Jungery M, Weatherall DJ, Parsons SF, Anstee DJ, and Tanner MJA (1982a) Glycophorin as a possible receptor for *Plasmodium falciparum*. Lancet *2*:947–950.

Pasvol G, Wainscoat JS, and Weatherall DJ (1982b) Erythrocytes deficient in glycophorin resist invasion by the malaria parasite *Plasmodium falciparum*. Nature *297*:64–66.

Peisach J, Blumberg WE, and Rachimelwitz EA (1975) The demonstration of ferrihemochrome intermediate in Heinz body formation following the reduction of hemoglobin A by acetylphenylhydrazine. Biochim. Biophys. Acta *393*:404–418.

Phillipson SW (1985) African archaeology. Cambridge: Cambridge University Press, pp. 113–147.

Ponnazhagan S, and Sarkar R (1992) Enzymes of the pentose phosphate pathway in glutathione-regulated membrane protection in beta-thalassaemia. Eur. J. Clin. Chem. Clin. Biochem. *30*:481–484.

Ponzetto-Zimmermann C, and Natta C (1981) Glutathione peroxidase activity in whole blood of patients with sickle cell anemia. Scand. J. Haematol. *26*:177–181.

Rachimelwitz EA, Peisach J, and Blumberg WE (1975) Studies on the stability of oxyhemoglobin A and its costituent chains and their derivatives. J. Biol. Chem. *246*:3356–3366.

Ranney HM, Lam R, and Rosenberg G (1993) Some properties of hemoglobin A2. Am. J. Hematol. *42*:107–111.

Roth EF, Friedman MJ, Ueda Y, Tellez I, Trager W, and Nagel RL (1978) Sickling rates of human AS red cells infected in vitro with *Plasmodium falciparum* malaria. Science *202*:650–652.

Roth E Jr, Raventos-Suarez C, Perkins M, and Nagel RL (1982) Glutathione stability and oxidative stress in *P. falciparum* infection in vitro: Responses of normal and G6PD deficient cells. Biochem. Biophys. Res. Commun. *109*:355–365.

Roth E Jr, Raventos-Suarez C, Rinaldi A, and Nagel R (1983) Glucose-6-phosphate dehydrogenase deficiency inhibits in vitro growth of *Plasmodium falciparum*. Proc. Natl. Acad. Sci. U.S.A. *80*:298–299.

Ruwende C, Khoo SC, Snow RW, Yates SNR, Kwiatkowski D, Gupta S, Warn P, Allsopp CEM, Gilbert SC, Peschu N, Newbold CI, Greenwood BM, Marsh K, and Hill AVS (1995) Natural selection of hemi- and heterozygotes for G6PD deficiency in Africa by resistance to severe malaria. Nature *376*:246–249.

Salhany JM, and Cassoly R (1989) Kinetics of *p*-mercurybenzoate binding of sulfhydryl groups on the isolated cytoplasmic fragment of band 3 protein. J. Biol. Chem. *265*:1399–1404.

Sallares R (1991) The Ecology of the Ancient Greek World. London: Duckworth.

Sauberman N, Fortier RL, Joshi W, Piotroskwi J, and Snyder LM (1983) Spectrin hemoglobin cross-linkages associated with in vitro oxidant hypersensitivity in pathologic and artifically dehydrated red cells. Br. J. Hematol. *54*:15–28.

Schluter K, and Drenckhahn D (1986) Co-clustering of denatured hemoglobin with band 3: Its role in binding of autoantibodies against band 3 to abnormal and aged erythrocytes. Proc. Natl. Acad. Sci. U.S.A. *83*: 6137–6141.

Schwarzer E, Turrini F, Ulliers D, Giribaldi G, Ginsburg H, and Arese P (1992) Impairment of macrophage functions after ingestion of *Plasmodium falciparum*-infected erythrocytes or isolated malarial pigment. J. Exp. Med. *176*:1033–1041.

Scott MD, Rouyer Fessard P, Lubin BH, and Beuzard Y (1990) Entrapment of purified alpha-hemoglobin chains in normal erythrocytes. A model for beta thalassemia. J. Biol. Chem. *265*:17953–17959.

Scott MD, Zuo L, Lubin BH, and Chiu DT (1991) NADPH, not glutathione, status modulates oxidant sensitivity in normal and glucose-6-phosphate dehydrogenase-deficient erythrocytes. Blood *77*:2059–2064.

Scott MD, van den Berg JJ, Repka T, Rouyer Fessard P, Hebbel RP, Beuzard Y, and Lubin BH (1993) Effect of excess alpha-hemoglobin chains on cellular and membrane oxidation in model beta-thalassemic erythrocytes. J. Clin. Invest. *91*:1706–1712.

Serjeantson S, Bryson K, Amato D, and Babona D (1977) Malaria and hereditary ovalocytosis. Hum. Genet. *37*:161–167.

Shaklai N, Yguerabide J, and Ranney HM (1977a) Interaction of hemoglobin with red blood cell membranes as shown by a fluorescent chromophore. Biochemistry *16*:5585–5592.

Shaklai N, Yguerabide J, and Ranney HM (1977b) Classification and localization of hemoglobin binding sites on the red blood cell membrane. Biochemistry *16*: 5593–5597.

Shaklay N, Sharma V, and Ranney HM (1981) Interaction of sickle cell hemoglobin with erythrocyte membranes. Proc. Natl. Acad. Sci. U.S.A. *78*:65–68.

Sharma R, and Premachandra BR (1991) Membrane bound hemoglobin as a marker of oxidative injury in adult and neonatal red blood cells. Biochem. Med. Metabol. Biol. *46*:33–44.

Shear HL (1993) Transgenic and mutant animal models to study mechanisms of protection of red cell genetic defects against malaria. Experientia *49*:37–42.

Shear HL, Roth EF Jr, Fabry ME, Costantini FD, Pachnis A, Hood A, and Nagel RL (1994) Transgenic mice expressing human sickle hemoglobin are partially resistant to rodent malaria. Blood *81*:222–226.

Sigerist HE (1965) Civilization and Disease. Chicago: Chicago University Press.

Simoes AP, van den Berg JJM, Roelofsen B, and Op den Kamp JAF (1992) Lipid peroxidation in *Plasmodium falciparum*-parasitized human erythrocytes. Arch. Biochem. Biophys. *298*:651–657.

Siniscalco M, Bernini L, Filippi G, Latte B, Meera Khan PM, Piomelli S, and Rattazzi M (1966) Population genetics of haemoglobin variants, thalassemia and glucose-6-phosphate dehydrogenase deficiency, with particular emphasis to the malaria hypothesis. Bull. World Health Organ. *34*:379–393.

Snyder LM, Leb L, Trainor J, Sauberman N, Liu SC, and Fortier NL (1983) Irreversible spectrin hemoglobin cross-linking in vivo: A marker for red cell senescence. Br. J. Hematol. *53*:379.

So Satta, Bernard O, and Long Y (1970) Résultats préliminaries d'une étude des rapports existant entre l'hémo-

globine E (HbE) et le paludisme au Cambodge. Nouv. Rev. Fr. Hematol. *10*:317–319.

Stuart-Macadam P (1992) Porotic hyperostosis: A new perspective. Am. J. Phys. Anthropol. *87*:39–47.

Thompson RB, Warrington R, Odom J, and Jim RTS (1962) Significance of haemoglobin S and C in Ghana. Br. Med. J. *i*:682–683.

Tilley L, Foley M, Anders RF, Dluzewski AR, Gratzer WB, Jones GL, and Sawyer WH (1990) Rotational dynamics of the integral membrane protein band 3, as a probe of the membrane events associated with *Plasmodium falciparum* infections of human erythrocytes. Biochim. Biophys. Acta *1025*:135–142.

Tilley L, Nash GB, Jones GL, and Sawyer WH (1991) Decreased rotational diffusion of band 3 in melanesian ovalocytes from Papua, New Guinea. J. Membr. Biol. *121*:59–66.

Trager W, and Jensen JB (1976) Human malaria parasites in continuous culture. Science *193*:673–675.

Turrini F, Mannu F, Arese P, Yuan J, and Low PS (1993) Characterization of the autologous antibodies that opsonize erythrocytes with clustered integral membrane proteins. Blood *81*:3146–3152.

van den Berg JJ, Op den Kamp JA, Lubin BH, Roelofsen B, and Kuypers FA (1992) Kinetics and site specificity of hydroperoxide-induced oxidative damage in red blood cells. Free Radic. Biol. Med. *12*:487–498.

van den Berg JJ (1994) Effects of oxidants and antioxidants evaluated using parinaric acid as a sensitive probe for oxidative stress. Redox Rep. *1*:11–21.

Vennerstrom JL, and Eaton JW (1988) Oxidants, oxidant drugs and malaria. J. Med. Chem. *31*:1269–1277.

Vernes AJM, Haynes JD, Tang DB, Dutoit E, and Diggs CL (1986) Decreased growth of *Plasmodium falciparum* in red cells containing hemoglobin E, a role for oxidative stress, and a sero epidemiological correlation. Trans. R. Soc. Trop. Med. Hyg. *80*:642–648.

Wallace WJ, Houtchens RA, Maxwell JC, and Caughey WS (1982) Mechanism of autoxidation for hemoglobins and myoglobins. Promotion of superoxide production by protons and anions. J. Biol. Chem. *257*: 4966–4977.

Waters AP, Higgins DG, and McCutchan TF (1991) *Plasmodium falciparum* appears to have arisen as a result of lateral transfer between avian and human hosts. Proc. Natl. Acad. Sci. U.S.A. *88*:3140–3144.

Waters AP, Higgins DG, and McCutchan TF (1993) The phylogeny of malaria: A useful study. Parasitol. Today *9*:246–250.

Weinstein IM, Spurling CL, Klein J, and Necheles TF (1954) Radioactive sodium chromate for the study of survival of red blood cells. III. The abnormal hemoglobin syndromes. Blood *9*:1155–1166.

Wernsdorfer WH (1994) Epidemiology of drug resistance in malaria. Acta Trop. *56*:143–156.

Wheaterall DJ (1987) Common genetic disorders of the red cell and the malaria hypothesis. Ann. Trop. Med. Parasitol. *81*:539–548.

Willcox M, Bjorkman A, and Brohult J (1983) Falciparum malaria and β-thalassaemia trait in northern Liberia. Ann. Trop. Med. Parassito. *77*:335–347.

Winterbourn CC (1990) Oxidative denaturation in congenital hemolytic anemias: The unstable hemoglobins. Semin. Hematol. *27*:41–50.

Wozencraft AO (1986) Damage to malaria infected erythrocytes following exposure to oxidant-generating systems. Parasitology *92*:559–567.

Yates SNR, Snow R, Allsopp CEM, Newton CRJC, Anstey N, Kwiatkowski D, Palmer D, Peschu N, Greenwood BM, Marsh K, Newbold CI, and Hill AVS (1993) α-thalassemia and malaria: Case control studies in East and West Africa. Proceedings of the British Society for Parasitology, 5th Malaria Meeting (abstract).

Yusa K, and Shikama K (1987) Oxidation of oxymyoglobin to metmyoglobin with hydrogen peroxide: involvement of ferryl intermediate. Biochemistry *26*:6684–6688.

Yuthavong Y, and Wilairat P (1993) Protection against malaria by thalassemia and haemoglobin variants. Parasitol. Today *9*:241–245.

Zulueta de J (1994) Malaria from prehistory to eradication. Parassitologia *36*:7–15.